Pheochromocytoma in dogs undergoing adrenalectomy

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Abstract: Pheochromocytoma is frequent in dogs and carries a guarded prognosis. Current histological criteria may not predict malignant behavior in dogs, similar to humans. In humans, characterization of tumors has been refined using the pheochromocytoma of the adrenal gland scaled score (PASS) and by immunohistochemistry. The study aim was to investigate PASS and immunohistochemical markers used in humans in 24 dogs with pheochromocytoma that underwent adrenalectomy. Dogs with pheochromocytomas were reviewed and tumors collected. Histological sections were evaluated to apply the PASS and were single-labeled for chromogranin A, Ki-67, COX-2, p53, BCL-2, c-erbB-2, vascular endothelial growth factor, and S100. Survival, age, and vascular and capsular invasion were compared for PASS and immunohistochemical markers; results of PASS were also compared for each marker. Associations between markers were tested. PASS and immunohistochemical markers did not differ for survival, age, and vascular and capsular invasion. Tumors showing BCL-2 expression in >50% cells had lower PASS than those with lower expression (PASS: 7 ± 2 vs 9 ± 2; P = .011). Tumors positive for S100 had higher PASS than those that were negative (PASS: 10 ± 2 vs 7 ± 2; P = .001). Results of the different markers were not associated. In conclusion, in the context of canine pheochromocytoma, PASS and the selected immunohistochemical markers are not associated with survival, age, or vascular or capsular invasion. The higher PASS in S100-positive tumors may indicate that pheochromocytomas developing morphologic changes acquire S100 expression. The significance of lower PASS in tumors with elevated BCL-2 expression is uncertain. Overall, the use of PASS and the present immunohistochemical markers may not be useful in dogs with pheochromocytoma.

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PHEOCHROMOCYTOMA IN DOGS UNDERGOING ADRENALECTOMY: AN EXPLORATORY INVESTIGATION OF 24 CASES

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Abstract

Pheochromocytoma is frequent in dogs and carries a guarded prognosis. Current histological criteria may not predict a malignant behaviour in dogs, similar to humans. In the latter, characterization of tumors has been refined by the "Pheochromocytoma of the Adrenal gland Scaled Score" (PASS) and by immunohistochemistry. The study aim was to investigate PASS and immunohistochemical markers used in humans in 24 dogs with pheochromocytoma that underwent adrenalectomy. Dogs with pheochromocytomas were reviewed and tumors collected. Histological sections were evaluated to apply the PASS and were single-labelled for chromogranin A, Ki-67, COX-2, p53, BCL-2, c-erbB-2, VEGF, S100. PASS and immunohistochemical markers were compared in light of survival, age, vascular and capsular invasion. Associations between markers were explored. PASS and immunohistochemical markers did not differ for any variable. Tumors showing BCL-2 expression in >50% cells had lower PASS than those with lower expression (7±2 vs. 9±2; *P*=0.011). Tumors positive for S100 had higher PASS than those negative (10±2 vs. 7±2; *P*=0.001). Associations between immunohistochemical markers were not identified. In conclusion, PASS and the selected immunohistochemical markers do not differentiate dogs with pheochromocytoma according to survival, age, vascular and capsular invasion. The lower PASS in dogs with elevated BCL-2 expression suggests that pheochromocytomas with high anti-apoptotic rate have few morphologic changes. The higher PASS in S100-positive tumors may indicate that pheochromocytomas developing morphologic changes acquire S100 expression. Overall, the use of PASS and the present immunohistochemical markers is of limited value in dogs with pheochromocytoma.

Keywords

Adrenal glands, dogs, immunohistochemistry, neoplasms, pheochromocytoma, scoring method, sustentacular cells.

Pheochromocytoma is a frequent neoplasia of the adrenal gland in dogs that arises from chromaffin cells. Most pheochromocytomas are monolateral and are usually identified as large masses occupying the entire gland, whereas less frequently as small nodules surrounded by a compressed rim of normal adrenal parenchyma.[3,5,10] Presenting complaints associated with pheochromocytoma are vague in the majority of dogs and during physical examination systemic arterial hypertension and cardiac arrhythmias may be identified.[3,5,10,14] Pheochromocytomas often invade the adrenal capsule and, through invasion of the adjacent tissues, they can encroach the caudal vena cava promoting the formation of large thrombi, which partially or totally occlude the vein leading to abdominal effusion.[20] Sudden weakness or death due to acute bleeding from the tumor or injured vessels is also described in affected dogs.[25]

From a clinical perspective, pheochromocytomas in dogs are usually considered as malignant when invasion through the capsule into adjacent tissues is present or when metastasis to distant sites is documented; they are locally invasive in 39-50% of cases and lead to distant metastases, including lymph nodes, liver, lungs, kidney, spleen and bone, in 13-24%.[3,10] With regard to prognosis, it is worth mentioning that life expectations of dogs with pheochromocytoma undergoing adrenalectomy are mostly associated with surgical outcome while it is unclear if local invasion and metastases are associated. Indeed, if dogs survive to surgery and are discharged from hospitalization, their median survival is longer than 3 years and most are lost to follow-up or die of another disease.[3] Further factors associated with a favorable outcome in dogs with pheochromocytoma are younger age and shorter duration of surgery.[11] In addition to the above studies, there are others on survival of pheochromocytoma but because they include both adrenal medullary and cortical tumors the results may be biased;[2,15-17] invasion of the caudal vena cava was variably associated with poor outcome in these investigations.

From a histological standpoint, morphologic criteria of tumor cells as defined by the world health organization (WHO) classification scheme may not predict a malignant behaviour in dogs with pheochromocytoma.[14] Similarly, prediction of pheochromocytoma behaviour in humans based on

histological assessment is debated and there is yet no single morphologic criterion that reliably anticipates a malignant course.[22] Hence, the diagnostic approach in humans with pheochromocytoma has been refined by the use of a histological score that takes into consideration different microscopic features and by the use of immunohistochemistry. Concerning the former, the "Pheochromocytoma of the Adrenal gland Scaled Score" (PASS), which is based on the combination of 12 different histologic parameters, has been developed and in different studies it was higher in lesions with malignant compared to benign course.[21,22] With regard to immunohistochemistry a number of markers have been investigated in pheochromocytoma in humans. Among them, a higher proliferation rate as assessed by Ki-67, an increased expression of cyclooxygenase-2 (COX-2), tumor suppressor gene product p53, anti-apoptotic product BCL-2, proto-oncogene product c-erbB-2, as well as vascular endothelial growth factor (VEGF) in tumor cells, and a decreased expression of protein S100 in sustentacular cells of the tumor were associated with worse prognosis.[7,19,22,23]

Because of the limited data currently available in dogs, the aim of the present study was to explore the possibility of application of PASS and of different immunohistochemical markers used in human medicine in a dog series affected by pheochromocytoma that underwent adrenalectomy. Surgical outcome, age and frequency of vascular and capsular invasion caused by the tumor were compared among the results of PASS and of immunohistochemistry. In addition, PASS was assessed in relation to immunohistochemical findings.

Materials and Methods

Dogs and inclusion criteria

From the pathology archive of the Department of Comparative Biomedicine and Food Science of the University of Padua, Italy, and from four surgeons (SN, VM, FM, GR) paraffin blocks of adrenal glands of dogs with a histological diagnosis of pheochromocytoma were retrieved. To be included in the study, pheochromocytoma had to be surgically excised by adrenalectomy and the tumor had to be chromogranin A positive or, if negative, had to show characteristic microscopic features of pheochromocytoma as described by the WHO Classification of Endocrine Tumors.[14] Data pertaining to signalement, including age, sex and breed, and affected adrenal gland, were retrieved from pathology records. Additional data were provided by the above surgeons or were requested to practitioners that submitted the adrenal sample for histology. For the latter purpose, a questionnaire was set up to acquire information concerning clinical and laboratory findings and presence of distant metastasis based on abdominal ultrasound, thorax radiography or computed tomography scan prior to surgery, tumor invasion of vessels such as caudal vena cava, renal vein or phrenico-addominal vein as identified by the radiologist with diagnostic imaging or by the surgeon during adrenalectomy, survival to discharge from hospitalization as well as long-term survival.

Histology

From each formalin-fixed paraffin-embedded specimen of canine pheochromocytoma, 4 µm-thick sections were obtained, stained with hematoxylin and eosin and evaluated using light microscopy (Olympus BX-40, Segrate, Italy). Microscopic criteria used by Thompson in 2002 to develop the PASS for human pheochromocytoma were applied to all cases,[22] including the presence of: i) large nests or diffuse growth (>10% of tumor volume), ii) central (middle of large nests) or confluent tumor necrosis (not degenerative change), iii) high cellularity, iv) cellular monotony, v) tumor cell spindling (even if focal), vi) mitotic figures >3 in 10 high power fields (HPF), vii) atypical mitotic figure(s), viii) extension into adipose tissue, ix) vascular invasion, x) capsular invasion, xi)

profound nuclear pleomorphism, and xii) nuclear hyperchromasia. The number of points assigned for the presence of each of the findings from i) to viii) was 2 and for each of the findings from ix) to xii) was 1; if findings were absent, 0 points were given. The PASS was calculated for each tumor as the sum of points to all 12 findings.

Immunohistochemistry

Additional 4 µm-thick sections were obtained from each specimen to perform immunohistochemistry using monoclonal or polyclonal antibodies with the following specificities: chromogranin A (dilution 1:50, clone rabbit anti-chromogranin A, Thermo Fisher Scientific, Chelmsford, MA), Ki-67 (dilution 1:50, clone MIB-1, Dako, Carpinteria, CA), COX-2 (dilution 1:700, purified mouse anti-COX-2, BD Biosciences, Milan, Italy), p53 (dilution 1:200, NCL-p53-CM1, Novocastra, New Castle, United Kingdom), BCL-2 (dilution 1:200, rabbit polyclonal IgG, Santa Cruz Biotechnology, Dallas, TX), c-erbB-2 (dilution 1:250, c-erbB-2 oncoprotein, Dako), VEGF (dilution 1:100, rabbit polyclonal IgG, Santa Cruz Biotechnology) and S100 (dilution 1:1000, rabbit polyclonal anti-S100, Dako).

Sections were mounted on Superfrost Plus microscope slides (Menzel, Braunschweig, Germany) and dried at 37°C for 30 minutes. All samples were tested with an automated immunostainer (Benchmark, Ventana Medical Systems, Tucson, AZ), which included dewaxing and rehydration, antigen retrieval, primary antibody incubation, antigen detection with an ultraView Universal diaminobenzidine (DAB) kit (Ventana Medical Systems) and counterstaining with Mayer's hematoxylin. Finally, slides were manually dehydrated through a graded series of alcohols and mounted (Eukitt mounting medium, Eukitt, Fort Washington, PA). Primary antibody dilutions were performed using a commercial antibody diluent (Ventana Medical Systems).

Different criteria were used to evaluate the slides for each specificity. In particular, for **chromogranin A** cytoplasmic positivity of neoplastic cells was scored based on the staining intensity as negative, mild and moderate-to-strong.[4]

For **Ki-67**, the percentage of neoplastic cells with nuclear positivity was counted in 1000 cells (counting 200 cells per 5 HPF);[4] to avoid overestimation of counts, neoplastic cells nearby or within vessels, necrotic and hemorrhagic areas were not considered.

For **COX-2**, cytoplasmic positivity of neoplastic cells was scored as negative (if 0-10% were positive) and positive (>10%).[19]

For **p53**, the percentage of neoplastic cells with nuclear positivity was scored as negative (if 0-10% were positive), mildly positive (11-50%) and moderately-to-strongly positive (>50%).[7]

For **BCL-2**, cytoplasmic positivity of neoplastic cells was scored as negative, positive in <50% cells and positive in >50% cells, as adapted from De Krijger and coworkers in 1999.[7]

For **c-erbB-2**, membrane positivity of neoplastic cells was scored as negative or positive.

For **VEGF**, the percentage of neoplastic cells with cytoplasmic positivity was scored as negative (if 0-10% cells were positive), mildly positive (11-50%) and moderately-to-strongly positive (>50%).[20]

Expression of **S100** was investigated in tumor cells and scored based on the staining intensity as negative, mild and moderate-to-strong.[22] Stellate or fusiform cells with hyperchromatic ovalar nuclei and S100 immuno-labelled cytoplasm were identified as sustentacular cells; in humans they are individually localized between tumor cells or around the nests.[4,24] The tumors were then considered as sustentacular cell-negative or sustentacular cell-positive based on the absence or presence of these cells. If positivity was documented, the percentage of sustentacular cells was counted in 1000 cells (counting 200 cells per 5 HPF) and also as average number of sustentacular cells in 10 HPF;[4,24] to avoid overestimation of counts, sustentacular cells nearby or within vessels, necrotic and hemorrhagic areas were not considered.

Statistical analysis

Information pertaining to signalement, clinical and laboratory findings, metastasis, tumor location, vascular invasion as identified by the radiologist or surgeon, histology and immunohistochemistry findings, as well as short and long-term outcome of dogs with pheochromocytoma were noted on a

spreadsheet. An exploratory investigation was carried out to compare PASS and each immunohistochemical marker between dogs that survived to discharge from hospitalization and those that died, between young and old dogs, between dogs with and without vascular invasion, and between dogs with and without invasion of the adrenal capsule. With regard to age, dogs were arbitrarily considered old if >10 years. With regard to vascular and capsular invasion, they were considered as present if identified by the pathologist. Because information regarding vascular invasion was also provided by the radiologist based on ultrasound or computed tomography of the abdomen, the analysis was also repeated by combining both microscopic and macroscopic identification of vascular invasion and by considering only the latter. In addition, the PASS was compared within the results of each immunohistochemical marker and associations between immunohistochemical findings were explored. The PASS was compared between groups using a t-test with Levene's test to assess equality of variances; because it includes assessment of vascular and capsular invasion,[22] each of the two criteria was excluded from the score to compare tumors with and without microscopic vascular or capsular invasion, respectively. Data were tested for normal distribution using the Shapiro-Wilk test and non-normal data were log-transformed and then analyzed using parametric tests. For each immunohistochemical marker the frequency was compared between groups with Fisher's exact test. Analyses were performed if at least 5 cases per group were available. Furthermore, Spearman correlation analysis was performed to investigate an association between PASS and immunohistochemical markers with quantitative data (i.e., the number of tumor cells positive for Ki-67 and the number of sustentacular cells). Lastly, survival to discharge from hospitalization was compared between pheochromocytoma-bearing dogs with and without vascular invasion (microscopic and macroscopic) as well as between those with and without capsular invasion using Fisher's exact test. Significance was set at $P<0.05$. Commercial software was used for statistical analysis (GraphPad Prism 4.0, San Diego, CA). All tissue sections were assessed by two pathologists (LC, SF) in a blinded fashion and inter-observer discrepancies were resolved by consensus.

Results

Dogs

Twenty-four dogs that underwent adrenalectomy and had a pheochromocytoma diagnosed based on histology were included. Their mean age was 11±3 years; age was not available in one dog. Gender was known in 23 dogs and 12 (52.2%) were intact male, 10 (43.5%) were spayed female and one (4.3%) was intact female. Breed was available for 20 cases, 12 (60%) were purebred and 8 (40%) were crossbred; among the former, 3 were Labrador Retriever, 2 were West Highland White Terrier and one each was American Cocker Spaniel, Boxer, Jack Russel Terrier, Lagotto Romagnolo, Miniature Schnauzer, Shit-Tzu and Siberian Husky.

Information regarding distant metastasis prior to adrenalectomy based on diagnostic imaging was available in 20 dogs and in none of them it was observed. Pheochromocytoma affected the left adrenal gland in 12 (60%) dogs and the right adrenal gland in 8 (40%) dogs; information was not obtained in 4 cases.

Ten (55.6%) dogs survived to discharge and 8 (44.4%) dogs died during surgery or hospitalization; in 6 dogs short-term outcome was unknown. For the 10 dogs with favorable outcome, 5 were still alive at the time of writing with a mean survival of 406±365 days and 5 died with a survival of 540±390 days; cause of death was unknown in 4 cases and was unrelated to pheochromocytoma in the latter (car accident).

Vascular invasion was documented by the radiologist or surgeon in 12 dogs (54.5%) and was not observed in 10 (45.5%) dogs; data were not achieved in 2 cases. Among the 12 dogs with vascular invasion, 5 had the caudal vena cava involved.

Information pertaining to clinical and laboratory results, as well as long-term survival were scant in the majority of dogs and therefore were not used for further investigation.

Histology

Hematoxylin and eosin-stained slides of the 24 dogs with pheocromocytoma were reviewed for morphologic assessment and to generate the PASS (Table 1).[22] None of the tumors showed capsule formation. Tumor cells breaking through the capsule of the adrenal gland, invading the surrounding tissue, was observed in 19 (79.2%) cases. Vascular invasion was identified in 17 (70.8%) pheocromocytomas; affected vessels were observed either within the adrenal capsule or beyond the tumor mass. In addition, invasion of the periadrenal adipose tissue by neoplastic cells was noted in 5 (20.8%) cases.

With regard to histological patterns, tumor cells were predominantly distributed in large nests or diffuse growth surrounded by thick acellular bands of fibrosis in 21 (87.5%) dogs with pheocromocytoma, while in the remaining 3 (12.5%) they were distributed in small nests. Diffuse or confluent necrosis was observed in 5 (20.8%) cases. The cellularity was high in 19 (79.2%) tumors and moderate in the other 5 (20.8%) cases. Tumor cell spindling was observed in only 1 dog.

In all 24 dogs the cytoplasm of tumor cells was uniformly granular and generally composed of eosinophilic granules, but it was occasionally light-eosinophilic or basophilic. Nuclear pleomorphism included enlarged nuclear size, irregular shape and bizarre forms and was noted in 20 (83.3%) cases. Cellular monotony was observed in 15 (62.5%) tumors while some cell variability was observed in the remaining 9 (37.5%). Nuclear hyperchromasia, identified as complete opacification and heavy nuclear deposition of chromatin, was observed in 10 (41.7%) tumors. In only one dog more than 3 mitoses in 10 HPF were counted; the same case showed atypical mitotic figures. The mean PASS of the 24 dogs was 8±2; the lowest score was 4 (1 dog), the highest was 12 (2 dogs).

Immunohistochemistry

Expression of chromogranin A was observed in all 24 dogs with pheochromocytoma, being mildly positive in 12 (50%) and moderately-to-strongly positive in 12 (50%) cases. The proliferation marker Ki-67 was negative in 11 (45.8%) tumors and positive in the other 13 (54.2%); of the latter, the mean percentage number of Ki-67 positive cells was 2.2±2.1% in 12 tumors and in the remaining case the amount was much higher, i.e. 22% (this case had capsular but not vascular invasion). The COX-2 was negative in 20 of 24 (83.3%) tumors and positive in the other 4 (16.7%) cases.

The tumor suppressor gene product p53 was evaluable in 21 of 24 (87.5%) dogs with pheochromocytoma and in 3 cases positivity was not assessed due to non-specific labelling; p53 was negative in 14 of 21 (66.7%) tumors, mildly positive in 3 (14.3%) and moderately-to-strongly positive in 4 (19%). The proto-onco gene product BCL-2 was expressed in all 24 tumors, being positive in <50% cells in 8 (33.3%) and positive in >50% cells in 16 (66.7%) cases. The proto-onco gene product c-erbB-2 was negative in 21 (87.5%) tumors and positive in the other 3 (12.5%). Expression of VEGF was negative in 13 (54.2%) tumors, mildly positive in 4 (16.7%) and moderately-to-strongly positive in 7 (29.1%).

Expression of S100 was evaluated among tumor cells, being negative in 11 of 24 (45.9%) cases, mildly positive in 7 (29.1%) and moderately-to-strongly positive in 6 (25%). Furthermore, the presence of sustentacular cells was investigated in 19 of 24 (79.2%) tumors, while in 5 cases their assessment was not possible due to excessive non-specific labelling or because the neoplastic tissue was strongly positive to S100 making sustentacular cells not distinguishable. Based on morphologic criteria and S100 immuno-labelling, positive sustentacular cells were observed in 6 (31.6%) of the 19 tumors; their mean percentage number was 3.6±1.9% and absolute number was 15.5±9.7.

Explorative analysis

The PASS did not differ between pheochromocytoma-bearing dogs that survived to discharge from hospitalization and those that died (9±2 vs. 8±2; *P*=0.229), between young and old dogs (9±2 vs. 8±2; *P*=0.110), between tumors with and without microscopic vascular invasion (9±2 vs. 7±1; *P*=0.058) and between those with and without invasion of the adrenal capsule (9±2 vs. 8±2; *P*=0.478). In addition, the PASS was not different between pheochromocytomas with or without macroscopic vascular invasion (9±3 vs. 8±2; *P*=0.449); the analysis combining microscopic and macroscopic vascular invasion was not performed due to the limited number of tumors without invasion.

Similarly to PASS, also the immunohistochemical markers were not different between groups (Table 2).

With regard to PASS and immunohistochemical markers, dogs with tumor cells showing BCL-2 expression in >50% cells had lower score than those with BCL-2 expression in <50% cells (7±2 vs. 9±2; *P*=0.011; Figure 1); as above mentioned, none of the dogs had tumors that were negative for BCL-2. In addition, dogs with mild or moderate-to-strong staining intensity of tumor cells for S100 had higher score than those with tumor cells negative for S100 (10±2 vs. 7±2; *P*=0.001; Figure 2). The PASS did not differ among the results of the other immunohistochemical markers. Correlations were not documented between PASS and the number of tumor cells positive for Ki-67 or the number of sustentacular cells. Associations between immunohistochemical markers were not identified.

The rate of survival to discharge from hospitalization did not differ between pheochromocytoma-bearing dogs with and without microscopic (9 of 10, 90% vs. 4 of 8, 50%; *P*=0.118) or macroscopic (4 of 10, 40% vs. 6 of 8, 75%; *P*=0.188) vascular invasion as well as between those with and without capsular invasion (8 of 10, 80% vs. 6 of 8, 75%; *P*=1.000). Age did not differ between survivors and non-survivors (10±3 years vs. 11±2 years; *P*=0.725).

Discussion

Pheochromocytoma is a frequent tumor of the adrenal gland that carries a guarded prognosis in dogs. Up to now few histological investigations have been performed in affected dogs and morphologic criteria did not appear to reliably predict a malignant course.[14] In order to better characterize this type of adrenal tumor in the present study the PASS, a score developed by Thompson for humans with pheochromocytoma that is based on 12 different morphological features,[22] was applied to dogs along with a number of immunohistochemical markers, including chromogranin A, Ki-67, COX-2, p53, BCL-2, c-erbB-2, VEGF and S100.[7,19,22,23] Unfortunately, their use was not able to differentiate between pheochromocytoma-bearing dogs that survived to discharge from hospitalization and those that died following adrenalectomy. Therefore, the scoring system and the immunohistochemical markers used in humans to differentiate patients with pheochromocytoma associated to a malignant course from those with a benign course do not provide survival information in affected dogs. Reasons for this discrepancy might be that in humans the reliability of PASS and of the listed immunohistochemical markers is not always clear-cut. Indeed, the PASS was not shown to be associated with outcome in some studies.[1,18] Furthermore, it is worth mentioning that in humans with pheochromocytoma the most frequently used cut-off of PASS to discriminate tumors with a malignant course is 4, with higher scores carrying a more guarded prognosis. In the present series only 1 dog had a PASS of 4 while the remaining 23 had higher scores, overall with a mean value of 8±2. Hence, another explanation for the lack of usefulness of PASS in dogs is that the intrinsic behaviour of pheochromocytoma in this species may differ from that in humans, with a more elevated degree of morphological abnormalities already present by the time of surgery. Whether this suggests that pheochromocytomas tend to be more malignant in dogs cannot be excluded. In support of this view it is remarkable that approximately half of our dogs died before discharge from hospitalization, while over 90% of pheochromocytomas in humans are benign and removed without complications.[6] Similarly to PASS, also some of the listed immunohistochemical markers, such as Ki-67, p53, BCL-2 and c-erbB-2, have not been

invariably associated with a malignant course in humans with pheochromocytoma.[7,18,21] Alternatively, the lack of association between PASS or immunohistochemical markers and survival is explained be the relatively low number of dogs included in this study; the limited number may have decreased the chance to detect significant differences. Finally, it is worth mentioning that in humans with pheochromocytoma an important outcome predictor is represented by the presence of distant metastasis or their development during follow-up; metastasis can occur 5 years, or longer, after surgery.[6] In our dog series, cases were treated by adrenalectomy if distant metastasis was not documented at admission and in most of those that survived to surgery it is unclear if metastasis developed during the subsequent months or years. Therefore, the dog group of the present investigation may be biased by the fact that none had known metastasis in the beginning and information was very limited during follow-up. The retrospective nature of the study did not allow to obtain a complete history of the dogs, in particular after surgical excision of the tumor.

Beside survival, the use of PASS and of the immunohistochemical markers did not yield any difference between pheochromocytomas with and without microscopic or macroscopic vascular invasion. The importance of vascular involvement is well known in dogs with pheochromocytoma, since renal veins and caudal vena cava are frequently affected. Macroscopic invasion was reported in half of the cases and 50% had the caudal vena cava infiltrated. Despite vascular invasion makes surgery more difficult, increasing the risk of acute and fatal bleeding,[17] in the present study pheochromocytoma-bearing dogs with and without vascular invasion had similar chance to survive to discharge from the hospital. Regardless of prognosis, pheochromocytomas with vascular invasion would be expected to have higher PASS or to have more frequent tumor cells positive for p53, BCL-2, c-erbB-2, VEGF or Ki-67. Actually, the dog with Ki-67 being positive in 22% of tumor cells had no vascular invasion. The absence of differences may suggest that other markers are more important in the pathogenesis of tumor spread. Differently from dogs, in humans with pheochromocytoma invasion of the inferior vena cava is very rare.[12] Thus, as above reported, it is possible that pheochromocytoma in dogs are more aggressive tumors than in humans.

The use of PASS and of the immunohistochemical markers did not yield any difference between pheochromocytomas with and without invasion of the adrenal capsule. As expressed for vascular invasion, differences would be expected also for capsular invasion. Although differences were not documented in dogs, in humans with pheochromocytoma it has been shown that capsular invasion is associated with higher proliferation rate of tumor cells based on Ki-67.[18]

Also, there were no differences for PASS and the immunohistochemical markers between young and old dogs. It is interesting to note that for pheochromocytomas a better outcome has been previously shown in younger dogs,[11] while in the present investigation age was not different between survivors and non-survivors. In humans with pheochromocytoma, those arising at a younger age are more likely to be malignant, to have a higher PASS and to be associated with a worse prognosis.[13] Further studies are needed to clarify the role of age in dogs with pheochromocytoma.

With regard to comparison of PASS for each immunohistochemical marker, it was observed that dogs with more than half of tumor cells showing expression of the anti-apoptotic product BCL-2 had lower score than those with less expression. This results is unexpected because in humans with pheochromocytoma both PASS and BCL-2 are expected to increased.[7,21,22] Whether an anti-apoptotic effect of BCL-2 decreases the chance of the tumor to acquire new morphological characteristics in dogs with pheochromocytoma remains to be elucidated. In addition, dogs with tumor cells positive for S100 had higher PASS than those with tumor cells negative for S100. This results is unclear, based on the notion that in humans pheochromocytoma cells with higher PASS are more likely to become S100 negative.[8] It may suggest that pheochromocytomas developing more morphologic changes also acquire S100 expression in dogs.

Notable, in dogs with pheochromocytomas it was possible to identify the sustentacular cells within tumors in approximately 30% of cases. Their presence was not associated to any other finding. As in humans, they were fusiform to stellate and individually localized between tumor cells or around the nests.[4,24] They are supposed to represent support elements, but also participate in the modulation

of the activity of the adrenal medulla, probably through paracrine effects.[9] In human medicine a higher number of sustentacular cells in malignant pheochromocytomas has been described,[23] but not in others.[4,24]

There are limitations to this study to be mentioned. The inclusion of dogs without metastasis at the time of surgery did not allow to achieve more information about potential associations between PASS or immunohistochemical markers and the disease course; however, in a clinical setting adrenalectomy is normally not proposed to owners if metastasis are not evident. Also, the absence of information about weight and size of the tumor prevented to study their possible associations with all the investigated features. Furthermore, in most cases the attending surgeon submitted only part of the tumor to pathology; hence, it cannot be excluded that some of the findings would have differed if the whole tumor was available for analysis.

In conclusion, in dogs with pheochromocytoma, survival, age, vascular and capsular invasion are not linked to the results of PASS and of the selected immunohistochemical markers. The lower PASS in dogs with elevated BCL-2 expression suggests that pheochromocytomas with high anti-apoptotic rate have few morphologic changes. The higher PASS in S100-positive tumors may indicate that pheochromocytomas developing morphologic changes acquire S100 expression. Overall, the use of PASS and of the present immunohistochemical markers is of limited value in dogs with pheochromocytoma.

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Table 1. Morphologic criteria, points assigned and calculated PASS in dogs with pheochromocytoma.

Case	Capsular invasion	Vascular invasion	Extension into fat	Large nests or diffuse growth	Central or confluent necrosis	High cellularity	Tumor spindling	Nuclear pleomorphism	Cellular monotomy	Nuclear hyperchromasia	Mitoses (>3/10 HPF)	Atypical mitoses	PASS
1	1	1	2	0	0	0	0	0	0	0	0	0	4
2	1	1	0	2	0	2	0	0	2	0	0	0	8
3	1	1	2	2	2	0	0	1	2	1	0	0	12
4	1	0	2	2	0	2	0	1	0	1	0	0	9
5	1	1	0	2	0	0	0	1	0	0	0	0	5
6	1	1	2	2	0	2	0	1	0	1	0	0	10
7	1	1	0	2	2	2	0	1	2	1	0	0	12
8	1	0	0	2	0	2	0	1	0	0	0	0	6
9	1	1	0	2	0	2	0	1	2	0	0	0	9
10	0	1	0	0	0	2	0	1	2	1	2	2	11
11	1	1	0	2	0	2	0	0	2	0	0	0	8
12	1	1	0	2	0	2	0	1	2	0	0	0	9
13	1	1	0	2	2	2	0	1	2	0	0	0	11
14	1	1	2	2	0	2	0	1	0	0	0	0	9
15	0	1	0	2	0	2	0	1	0	1	0	0	7
16	0	1	0	2	0	2	0	1	2	1	0	0	9
17	1	1	0	2	0	2	0	1	2	1	0	0	10
18	0	0	0	2	2	0	0	1	0	0	0	0	5
19	1	0	0	2	0	2	0	1	2	0	0	0	8
20	1	0	0	2	0	2	0	0	2	0	0	0	7
21	1	1	0	0	0	2	0	1	2	1	0	0	8
22	0	0	0	2	0	0	2	1	2	0	0	0	7
23	1	1	0	2	0	2	0	1	2	1	0	0	10
24	1	0	0	2	2	2	0	1	0	0	0	0	8

Points assigned for the presence of each finding is 1 or 2; if absent, 0 points are given.[22] HPF, high power fields; PASS, Pheochromocytoma of the Adrenal gland Scaled Score.

Table 2. Immunohistochemical markers in dogs with pheochromocytoma and comparison between groups.

		Chromogranin A	**Ki-67**	**COX-2**	**p53**	**BCL-2**	**c-erbB-2**	**VEGF**	**S100**	**S100**
		(moderately-to-strongly positive)	(positive)	(positive)	(mildely or moderately-to-strongly positive)	(positive in >50%)	(positive)	(mildely or moderately-to-strongly positive)	(mildely or moderately-to-strongly positive)	(positive sustentacular cells)
Survival to discharge	yes	6/10 (60%)	5/10 (50%)	3/10 (30%)	4/10 (40%)	6/10 (60%)	0/10 (0%)	4/10 (40%)	6/10 (60%)	2/8 (25%)
	no	3/8 (37.5%)	5/8 (62.5%)	1/8 (12.5%)	3/8 (37.5%)	5/8 (62.5%)	1/8 (12.5%)	3/8 (37.5%)	4/8 (50%)	3/6 (50%)
	P	0.637	0.664	0.588	1.000	1.000	1.000	1.000	1.000	0.580
Age (years)	>10	5/11 (45.5%)	6/11 (54.5%)	1/11 (9.1%)	3/9 (33.3%)	8/11 (72.7%)	1/11 (9.1%)	4/11 (36.4%)	6/11 (54.5%)	1/8 (12.5%)
	≤10	6/12 (50%)	6/12 (50%)	3/12 (25%)	4/11 (36.4%)	7/12 (58.3%)	1/12 (8.3%)	6/12 (50%)	6/12 (50%)	5/11 (45.5%)
	P	1.000	1.000	0.590	1.000	0.667	1.000	0.680	1.000	0.177
Vascular invasion (microscopic)	yes	9/17 (52.9%)	8/17 (47.1%)	4/17 (23.5%)	6/15 (40%)	11/17 (64.7%)	3/17 (17.7%)	9/17 (52.9%)	8/17 (47.1%)	4/14 (28.6%)
	no	3/7 (42.9%)	5/7 (71.4%)	0/7 (0%)	1/6 (16.7%)	5/7 (71.4%)	0/7 (0%)	2/7 (28.6%)	5/7 (71.4%)	2/5 (40%)
	P	1.000	0.386	0.283	0.613	1.000	0.529	0.386	0.386	1.000
Vascular invasion (macroscopic)	yes	5/12 (41.7%)	6/12 (50%)	2/12 (16.7%)	6/10 (60%)	8/12 (66.7%)	1/12 (8.3%)	4/12 (33.3%)	4/12 (33.3%)	3/11 (27.3%)
	no	6/10 (60%)	6/10 (60%)	2/10 (20%)	1/10 (10%)	6/10 (60%)	1/10 (10%)	5/10 (50%)	7/10 (70%)	3/7 (42.8%)
	P	0.669	0.691	1.000	0.057	1.000	1.000	0.666	0.198	0.627
Capsular invasion	yes	8/19 (42.1%)	10/19 (52.6%)	3/19 (15.8%)	7/16 (43.8%)	4/17 (23.5%)	2/19 (10.5%)	9/19 (47.4%)	9/19 (47.4%)	6/18 (33.3%)
	no	4/5 (80%)	3/5 (60%)	1/5 (20%)	0/5 (0%)	3/5 (60%)	1/5 (20%)	2/5 (40%)	4/5 (80%)	n.a.
	P	0.317	1.000	1.000	0.124	0.274	1.000	1.000	0.327	n.a.

P, probability-value; n.a., not available because the number of cases was less than 5 in one group.

Figure captions

Figure 1. Scatter plot of PASS in pheochromocytomas with BCL-2 expressed in >50% cells and in <50% cells. The horizontal line is the mean value.

Figure 2. Scatter plot of PASS in pheochromocytomas with mild or moderate-to-strong staining intensity of tumor cells for S100 (positive) and with negative results. The horizontal line is the mean value.